Robert M. Campbell*
Jacob Bongers
Arthur M. Felix
Hoffmann–La Roche, Inc.
Roche Research Center
Nutley, New Jersey 07110

Rational Design, Synthesis, and Biological Evaluation of Novel Growth Hormone Releasing Factor Analogues

Since its initial discovery in 1982, growth hormone-releasing factor (GRF) has been the subject of intense investigation. This interest was prompted by the potential application of GRF for stimulating growth in dwarf humans and for performance enhancement in livestock. Substantial research has been focused upon the development of potent, long-acting analogs as therapeutics. Herein is described a summary of the cumulative efforts of various laboratories endeavoring in this quest. The rationale utilized in GRF analog development is discussed: 1) determination of bioactive core, 2) evaluation of secondary structure, and 3) elucidation of degradation pathways (chemical and enzymatic). Using this information, several series of linear (unnatural and natural sequence) and cyclic GRF analogs were designed, synthesized, and evaluated. Stimulated by the constraints of commercial production, innovative, alternative methods of synthesis were explored: solid-phase, solution-phase, enzymatic, and recombinant. To date, the most promising candidate for drug development is [His^1, Val^2, Gln^8, Ala^{15}, Leu^{27}]-hGRF(1-32)-OH. This natural sequence analog, consisting of rodent and human sequences, incorporates the bioactive core, preferred secondary structure, resistance to chemical and enzymatic degradation; with the added benefit of amenability to large-scale recombinant synthesis.

INTRODUCTION

In 1982, three novel peptides consisting of 37 (free carboxy), 40 (free carboxy), and 44 (amidated) amino acid residues were isolated from a human pancreatic tumor. These peptides were identical from the amino terminus, and each potently stimulated the release of growth hormone (GH) from the pituitary gland.[1,2] Shortly after, the major endogenous form of this growth hormone releasing factor (GRF) was purified from hypothalamic tissue and found to be GRF(1–44)-NH_2 (see Refs. 3 and 4; Figure 1). The human GRF(1–44)-NH_2 (hGRF) sequence was also confirmed by expression cloning and sequencing of the cDNA.[5,6] Primary structures of hypothalamic GRF, ranging from 42 to 45 amino acids in length, were subsequently elucidated for cattle,[7] sheep and goat,[8] pig,[9] rat,[10] mouse,[11,12] carp,[13] and salmon[14] (Figure 1). Human, cattle, sheep, goat, and pig GRF are highly homologous (86–93% sequence identity), each being comprised of 44 amino acid residues with an amidated carboxy terminus. In contrast, rat, mouse, carp, and salmon GRF are less homologous to their mammalian counterparts (40–74% sequence identity) and contain 43, 42, 45, and 45 residues, respectively, with free carboxy termini.

Not unexpectedly, it was found that hGRF

* To whom correspondence should be addressed at Department of Inflammation/Autoimmune Diseases, Hoffmann-La Roche, Inc., Nutley, NJ 07110-1199.

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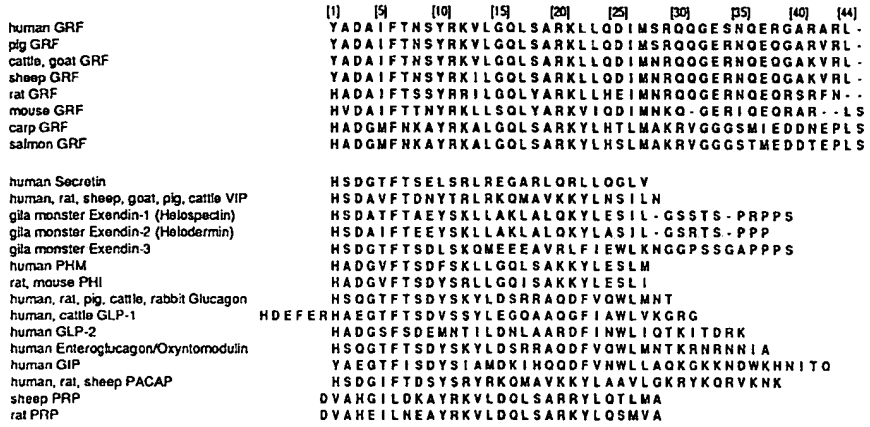


FIGURE 1 Amino acid sequences[13,15] of vertebrate GRF, secretins, vasoactive intestinal polypeptides (VIP), PHI/PHM, exendins, glucagons, glucagon-like peptides (GLP), enteroglucagons/oxyntomodulins, gastric-inhibiting peptides (GIP), pituitary adenyl cyclase activating polypeptides (PACAP), and PACAP-related peptides (PRP). Sequences were aligned using theoretical deletions to maximize homology. Standard single-letter abbreviations are used for amino acids (IUPAC-IUB Commission on Biochemical Nomenclature): A: Ala; C: Cys; D: Asp; E: Glu; F: Phe; G: Gly; H: His; I: Ile; K: Lys; L: Leu; M: Met; N: Asn; P: Pro; Q: Gln; R: Arg; S: Ser; T: Thr; V: Val; W: Trp; Y: Tyr; (–): gap/deletion.

could directly stimulate pituitary GH release from a number of higher vertebrates (e.g., human, monkey, cattle, goat, sheep, pig; for review, see Ref. 15). Therefore, it was a likely presumption that GRF could be utilized to enhance growth in both dwarf (i.e., GH-deficient) children and domestic animals. In the case of domestic livestock, an added benefit of GRF would be a leaner carcass (decreased fat, increased muscle) for human consumption. Prompted by the clinical and veterinary potential of GRF, concerted efforts were made by various laboratories to develop potent, long-acting analogues. Herein is described, from a historical perspective, both academic and industrial approaches to the design, synthesis, and biological evaluation of linear and cyclic GRF analogues.

RATIONAL DRUG DESIGN

The goal of producing potent, long-acting bioactive peptides requires a coordinated, rational, and interdisciplinary approach. In the case of GRF, the general parameters of importance were (1) to determine the minimal sequence that retains intrinsic biological activity (i.e., the "bioactive core"), (2) to determine its secondary structure (i.e., to develop a model to predict the theoretically preferred bioactive conformation), (3) to ascertain modes and pharmacokinetics of decomposition, and (4) to selectively replace amino acid residues within the bioactive core that might increase receptor affinity and/or inhibit degradation. Inherent within the design pathway is the requirement for in vitro and in vivo models by which to quantitate biological activity and half-life. Once a lead candidate was selected for commercial application, novel synthetic strategies would be required to economize the process.

GRF BIOACTIVE CORE

Due to the high degree of sequence conservation in the proximal 29 residues of GRF in various species (55–100%; Table I), it was postulated that GRF(1–29) might constitute the bioactive core of the molecule. Utilizing the strategy of C-terminal deletion (i.e., determining the effect of removing one amino acid at a time from the C-terminus), Ling and coworkers[16] established that hGRF(1–29)-NH_2 was indeed the shortest peptide that retained substantial intrinsic GH-releasing activity in vitro. This finding was further confirmed by the equivalency of hGRF(1–29)-NH_2 to hGRF(1–44)-NH_2 in receptor-binding[17] and in vivo GH-releasing assays

Table I Amino Acid Sequence Identities Between Growth Hormone Releasing Factors from Various Species[a]

	Relative to Human GRF(1–44)-NH_2	Relative to Human GRF(1–29)-NH_2
Human GRF(1–44)-NH_2	100% (44/44)	100% (29/29)
Pig GRF(1–44)-NH_2	93.2% (41/44)	100% (29/29)
Goat GRF(1–44)-NH_2	88.6% (39/44)	96.6% (28/29)
Cow GRF(1–44)-NH_2	88.6% (39/44)	96.6% (28/29)
Sheep GRF(1–44)-NH_2	86.4% (38/44)	93.1% (27/29)
Rat GRF(1–43)-OH	65.9% (29/44)	72.4% (21/29)
Mouse GRF(1–42)-OH	59.1% (26/44)	62.1% (18/29)
Carp GRF(1–45)-OH	40.9% (18/44)	55.2% (16/29)
Salmon GRF(1–45)-OH	40.9% (18/44)	55.2% (16/29)

[a] GRF sequences and suggested alignments are derived from Figure 1.

(humans: Refs. 18 and 19; mice: Ref. 20; sheep: Ref. 21; cattle: Refs. 22 and 23; pigs: Refs. 23 and 24). The N-terminus of hGRF is critical for activity, since deletion of [Tyr^1]- or [Tyr^1, Ala^2]- reduces biological potency by several orders of magnitude.[2,17] The relative importance and specificity of position 1 is emphasized by its intolerance for most amino acid substitutions (e.g., Phe^1, Trp^1, Ala^1, 3MeHis[1], OMeTyr[1], or NAcTyr[1]).[25] Only modest replacements, such as desNH_2Tyr^1 or $NMeTyr^1$,[17,20,26–28] do not hinder GRF binding and activity.

GRF SECONDARY STRUCTURE

Based upon sequence homology, GRF is a member of a large peptide superfamily that includes secretin, PHI/PHM, VIP, glucagon, GIP, and PACAP (Figure 1).[15,29] A predominantly α-helical conformation is manifested by various members of this superfamily, e.g., glucagon,[30,31] secretin,[31,32] VIP,[31,33] and PACAP.[34] In concordance, hGRF is also largely α-helical.[35–38]

Secondary structure data [CD, two-dimensional (2D) nmr spectra] from our laboratories indicate an α-helical character between hGRF residues 4–29 in 75% methanol/water (Figure 2) and between residues 9–14 and 24–28 in water.[37,38] Similarly, nmr data from Clore and co-workers[35,36] suggested that hGRF was α-helical between residues 6–13 and 16–29 in 30% TFE/water. Therefore, most nonconservative substitutions between sequenced GRF species[15] are found outside of the predicted helix (beyond residue 30; see Figures 1 and 2) apart from the N-terminal residues; the majority of the putative bioactive core is helical. Incorporating this information, it was hypothesized that amino acid substitutions stabilizing and/or extended the α-helix might increase the affinity of GRF for its recep-

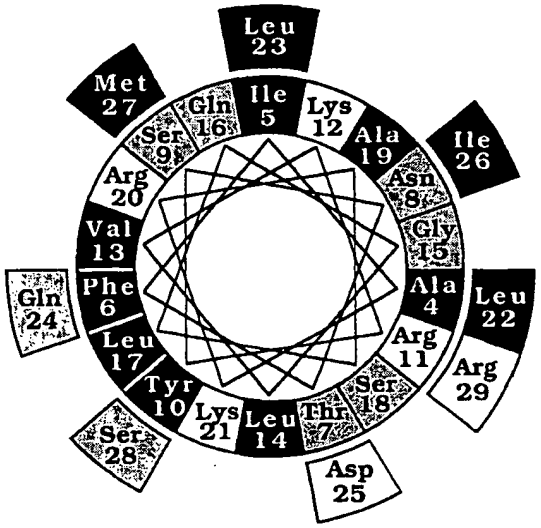


FIGURE 2 Edmundson wheel projection of human/pig GRF(4–29) in a hypothetical α-helical conformation (turn frequency $\approx$ 4 residues) based on 2D nmr solution structure data[37,38] in 75% methanol/water. Although a continuous helical span from residues 4–29 is described, breaks in the helix [between residues 13–16 in 30% trifluoroethanol (TFE)/water and 14–24 in 100% water] have been observed in more aqueous environments.[35–38] Hydrophobic residues are black (■), neutral residues shaded (□), and charged residues white (□).

Table II Potencies and Receptor Binding Affinities of hGRF Analogues Relative to hGRF(1–44)-NH_2 in vitro[a]

hGRF Analogue	Relative Potency[b]	Relative Affinity[c]	Plasma Half-Life[d]
hGRF(1–44)-NH_2 ($hGRF_{44}$)	1.00	1.00	7.5 min
[Met(O)^{27}]-$hGRF_{44}$	0.30	0.17	
hGRF(1–29)-NH_2 ($hGRF_{29}$)	0.71	0.91	6.5 min
[Met(O)^{27}]-$hGRF_{29}$	0.32	0.17	
[desNH$_2$Tyr^1]-$hGRF_{29}$	1.24	0.82	
[D-Ala^2]-$hGRF_{29}$	1.81	1.98	
[Ala^{15}]-$hGRF_{29}$	3.81	3.60	8.5 min
[desNH$_2$Tyr^1, Ala^{15}]-$hGRF_{29}$	4.04	4.50	30 min
[D-Ala^2, Ala^{15}]-$hGRF_{29}$	4.86	4.87	32 min
[desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-$hGRF_{29}$	4.70	5.17	42 min
[Sar^{15}]-$hGRF_{29}$	< 0.001	< 0.001	
hGRF(3–44)-NH_2	< 0.001	< 0.001	
hGRF(3–29)-NH_2	< 0.001	< 0.001	
hGRF(12–29)-NH_2	< 0.001	< 0.001	
hGRF(13–29)-NH_2	< 0.001	< 0.001	
hGRF(21–29)-NH_2	< 0.001	< 0.001	
hGRF(22–29)-NH_2	< 0.001	< 0.001	
hGRF(1–11)-OH	< 0.001	< 0.001	
hGRF(1–20)-NH_2	< 0.001	< 0.001	
Secretin, glucagon, VIP, PHI [1–27]	< 0.001	< 0.001	

[a] Data in part from Campbell et al.[17].

[b] GH-releasing potency relative to hGRF(1–44)-NH_2; determined using cultured rat pituitary cells exposed to 3.1 → 400 pM HGRF analogue for 4 h, 37°C.

[c] Binding affinities were determined by competitive displacement of [His^1, ^{125}I-Tyr^{10}, Nle^{27}]-hGRF(1–32)-NH_2 from rat pituitary homogenates (60 min, 24°C).

[d] Stability was examined by incubating GRF analogues in porcine plasma for 4 h, 37°C, and measuring amount remaining by HPLC.

tor. Ensuing experiments lend support for this hypothesis. Substitution of Sar^{15}, a helix-breaking residue, for native Gly^{15} abolished the receptor binding affinity and GH-releasing activity of hGRF(1–29)-NH_2 (Table II).[17,39] Conversely, substitution of Ala^{15}, a helix-forming residue, significantly improved the receptor binding affinity and GH-releasing activity of hGRF(1–29)-NH_2.[17,20,39]

It was also reported that specific regions of peptides assumed amphiphilic secondary structures under physiological conditions, such as might be conducive to ligand–cell membrane receptor intercalation.[40] Theoretically, selective amino acid replacements could allow for improved ligand insertion into the amphipathic bilayer of the pituitary cell membrane. Improved stability/rigidity of the α-helix imparted by these substitutions (via increased intermolecular interactions) might increase the "tightness of fit" or affinity for the GRF receptor. Hence, the model of "preferred bioactive conformation" was expanded to incorporate the proposition of a stabilized, amphiphilic α-helix being optimal for GRF receptor interactions.

DECOMPOSITION OF GRF

Nonenzymatic Degradation Pathways

GRF may decompose nonenzymatically by way of oxidation. GRF is susceptible to methionine sulfoxide formation at position 27 [Met^{27} → Met(O)^{27}],[41,42] resulting in greatly reduced receptor affinity/bioactivity.[17,42] Apart from its propensity to oxidize, Met^{27} precludes the use of cyanogen bromide for fusion protein cleavage in recombinant DNA synthesis. These situations may be alleviated by substitution of other hydrophobic residues such as Leu, Ile (recombinant or chemical synthesis), or Nle (chemical synthesis only), which maintain the regional amphiphilic character, are not oxidized and retain activity.[39,41,43–47] The ac-

ceptability of such replacements indicates that strict conservation of Met^{27} is not necessary for GRF receptor binding.

Although transpiring at a considerably slower rate ($T_{1/2} \approx$ 100–200 h) than enzymatic degradation ($T_{1/2} \approx$ 7–18 min for DPP-IV; see below), hGRF and analogues[45,48–50] have been shown to undergo deamidation and isomerization (Asn^8 conversion to Asp^8 and β-Asp^8) in aqueous buffer in vitro. This mode of Asn^8 rearrangement is associated with substantial loss (>95%) in bioactivity,[45,48–50] presumably due to disruption of the α-helix.[51] Asp^3 degradation to β-Asp^3 may also occur with a somewhat less severe reduction in bioactivity (70–80%).[48,50] Deamidation of either Asp^{25} or Asn^{35} was not seen in vitro, presumably due to steric or conformational hindrance.[50] It has been inferred from data using Asn^{28}-substituted hGRF(1–32)-NH_2 analogues[45] that Asn^{28} (found in cattle, goat, sheep, rat, and mouse GRF sequences) might indeed deamidate. In swine studies using sustained release formulations of a hGRF analogue, only Asn^8 deamidation/isomerization (not Asp^3, Asp^{25}, or Asn^{35}) was observed following several weeks of subcutaneous implantation.[44] Hence, acceptable position 8 replacements would be required to prevent degradation (i.e., deamidation/isomerization) of hGRF in aqueous environments over prolonged time periods (e.g., relevant to formulation and sustained release delivery).

Enzymatic Degradation Pathways

Human,[12,52] porcine,[53] bovine,[54] and rat[55] GRF are rapidly degraded in plasma by dipeptidylpeptidase IV enzyme (DPP-IV). DPP-IV hydrolyzes the peptide bond between residues Ala^2 and Asp^3, dramatically reducing GRF receptor binding and GH-releasing activity.[2,17,52] Since human GRF analogues with His^1-(Ala^2) substitution,[43,44,56,57] as well as other GRF family members with His^1-Ala^2-N-termini (e.g., GIP, GLP-1, PHM; see also Figure 1),[58] are rapidly degraded by DPP-IV in vitro, carp and salmon GRF (containing His^1-Ala^2-) likely would also be susceptible to DPP-IV cleavage. Contrary to all other known sequences, the N-terminus of mouse GRF is His^1-Val^2.[11,12] The significance of this aberrant N-terminus was not initially realized until instigated by a need for "natural sequence" recombinant GRF analogues (see Natural Amino Acid Composition).

Albeit to a lesser extent, human[59,60] and rat[55] GRF are subject to degradation by trypsin-like endopeptidases in plasma (between basic residues: $Arg^{11,20}$ and $Lys^{12,21}$). Proteolytic cleavage between these residues effectively inactivates GRF.[17,55] One laboratory did note that hGRF analogues were proteolytically cleaved in rat plasma between Thr^7 and Asn^8/Ser^8.[60,61] However, neither Thr^7-Asn^8 (human, swine, or bovine GRF) nor Thr^7-Ser^8 (rat GRF) proteolysis was observed in incubations with human, swine, or bovine plasma.[53,54,59] Therefore, long-acting GRF analogues would require amino acid substitutions at the N-terminus to prevent recognition by DPP-IV; and possibly also at residues Arg^{11}, Lys^{12}, Arg^{20}, and/or Lys^{21} to avoid trypsin-like cleavage.

LINEAR GRF ANALOGUES

Unnatural Amino Acid Composition

Shortly after the sequencing of hGRF, a number of laboratories expeditiously set out to synthesize linear analogues with enhanced potency and stability profiles. Early attempts utilizing D-amino acid substitution were generally unsuccessful, yielding mostly analogues with unimproved or reduced potency in vitro (GH release: Refs. 25, 41, and 62; adenyl cyclase activity: Ref. 63; and in the case of D-Arg^2 substitution, GRF antagonists: Refs. 62 and 64). [D-Ala^2]-hGRF(1–29)-NH_2 was initially described as being 50-fold more potent than hGRF in the rat in vitro[65] and in vivo.[66,67] This potency appeared to be an overestimation and was later revised to $\approx$ 2–3-fold in vitro (rat: Ref. 17, 39, 62, and 68) and 1–2-fold in vivo (cattle: Ref. 22; human: Refs. 18 and 69; swine: R. Campbell, E. Heimer, and A. Felix, unpublished observations). The advantage of D-Ala^2 (as well as D-Asp^3) substitution lay in its ability to inhibit DPP-IV mediated proteolysis.[56,59] Substitution of desNH$_2$$Tyr^1$- (one of the few acceptable replacements at position 1), in combination with D-Ala^2, gave rise to hGRF analogues that were resistant to DPP-IV proteolysis.[53,57,59]

To maximize the benefit of increased biological half-life provided by [desNH$_2$$Tyr^1$, D-$Ala^2$] substitution, the optimal hGRF analogue would need to be inherently potent (i.e., as assessed by GH-releasing activity in vitro). Based upon an Edmundson helical wheel projection of hGRF(4–29) (Figure 2), it was thought that hydrophobic amino acid replacement of Gly^{15} might improve the amphipathic nature of the helix, ligand–receptor interac-

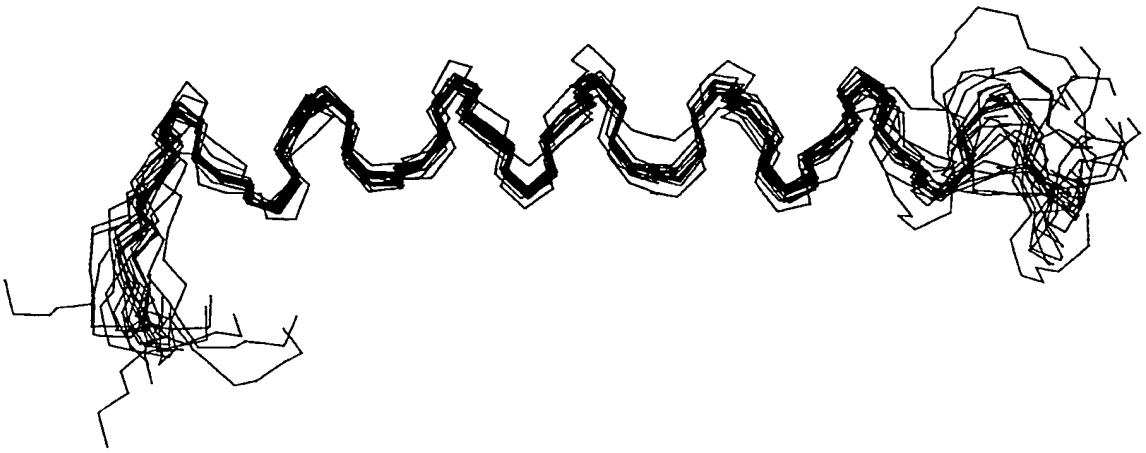


FIGURE 3 Superposition of structures of [Ala^{15}]-hGRF(1–29)-NH_2 obtained from evaluation of nmr data in 75% methanol, pH 6.0 by constrained molecular dynamics and minimization calculations.

tion, and activity. Moreover, selection of helix-forming residues (i.e., displaying increased intramolecular hydrogen bonding) would additionally stabilize the secondary structure. Indeed, position 15 substitution with a helix-stabilizing, hydrophobic residue such as Leu, Ala, or Aib (for native Gly^{15}) GH-releasing potency is nearly equal to 4-fold in vitro.[39] In vivo, [Ala^{15}]-hGRF(1–29)-NH_2 is 5–6-fold more potent than its full-length parent, hGRF(1–44)-NH_2 (mouse: Ref. 20; swine and cattle: R. Campbell, R. Miller, E. Heimer, and A. Felix, unpublished observations). In accordance with our theoretical model, conformational analysis (CD and molecular dynamics calculations based on nuclear Overhauser effect (NOE) derived distance constraints) confirmed that [Ala^{15}]-hGRF(1–29)-NH_2 has a preferred conformation that includes enhancement of a central α-helical segment comprising residues 4–26 (solution structure in 75% methanol/water: Refs. 38 and 70; Figure 3).

Combination of [Ala^{15}]- with [desNH_2Tyr^1, D-Ala^2] substitution produced synergistic effects: an analogue that was intrinsically potent ($\approx$ 5-fold that of hGRF) and resistant to plasma degradation ($\approx$ 4-fold that of hGRF) in vitro: [desNH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 (Table II).[12,17,27] [desNH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 was subsequently determined to be approximately 10–15-fold more potent than hGRF in vivo (mouse: Ref. 20; swine and cattle: R. Campbell, R. Miller, E. Heimer, and A. Felix, unpublished observations). The high in vivo activity of [des-NH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 was particularly significant in that it represented the composite effects of receptor affinity, chemical/enzymatic degradation, and metabolic clearance (e.g., sequestration and/or metabolism by tissues).

Enzymatic Semisyntheses of GRF and Potent Analogues

Rationale for Enzymatic Synthesis. Designing and operating an efficient and economical large-scale synthesis of a peptide is often a path strewn with hazards. Peptides of intermediate length (ca. 15–50 residues), such as GRF and analogues, present a particularly vexing problem as they are often too large to be prepared economically by conventional solid-phase methods and too small for a successful recombinant DNA synthesis. The latter problem occurs when the host cell marks the desired peptide product for destruction as a foreign antigen rather than secreting or storing it. As to the former problem, while the solid-phase method has enabled the synthesis of a multitude of analogues for structure–activity studies, scaling up to multikilogram levels presents special difficulties.

During the course of our studies, we were confronted with developing large-scale syntheses of the parent hGRF(1–44)-NH_2 intended for the treatment of growth disorders in children,[71] and the superpotent GRF analogue, [desNH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2,[39] to eventually be produced in multikilogram amounts as an animal

health product for the enhancement of growth performance in domestic livestock.[23,24,72] The high cost and inherent difficulties of producing either of these peptides by solid-phase methods on a large scale led us to consider recombinant DNA technology. The problem of antigenicity to the host cell can be circumvented by expressing the target peptide as part of a fusion protein, i.e., packaged with a larger properly processed carrier protein, and then chemically or enzymatically liberating it from the fusion protein after harvesting from the cell culture. However, both of the target peptides—hGRF(1–44)-NH_2 and [desNH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2—contain structural features that are not coded nor expressed in existing recombinant cell lines (i.e., the amidated C-termini and, in the latter analogue, the unnatural N-terminal amino acids, desNH_2Tyr^1 and D-Ala^2).

We pursued a combined recombinant/enzymatic approach whereby C-terminal amidation and attachment of the desNH_2Tyr-D-Ala moiety would be performed via selective enzyme catalyzed modifications to recombinant precursors. In the case of the GRF analogue, we planned to attach desNH_2Tyr-D-Ala using a proteolytic enzyme to synthesize a new peptide bond rather than cleaving an existing peptide bond (as in the case of the prohormone precursors). The proteolytic enzymes are naturally suited for use in chemical synthesis for the selective hydrolysis of peptide bonds and esters. Use of the proteases as catalysts for peptide bond formation is generally more difficult and the subject of current research (for reviews, see Refs. 73–80).

In this section, we review studies of several enzymatic C-terminal amidations of unprotected GRF precursor peptides that include several protease catalyzed acyl transfers and a naturally occurring α-amidating enzyme catalyzed oxidative route employed in vitro. We will also review a protease catalyzed 3-mer + 26-mer segment condensation for the incorporation of unnatural amino acid residues into the N-terminus of a recombinant precursor of the superpotent GRF analogue and a 33-mer + 11-mer segment condensation employed in a convergent solid-phase/enzymatic synthesis of the parent hGRF. Although these studies were undertaken with the very narrow aim of developing recombinant/enzymatic syntheses of hGRF(1–44)-NH_2 and [desNH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, we have made several observations about these types of enzymatic systems that are intended to be of general interest to the reader.

Preparative Enzymatic C-Terminal Amidations of GRF Precursors. *The α-Amidating Enzyme.* The amidated C-terminus of peptide hormones and other bioactive peptides often affords full receptor binding affinity and/or protection from carboxypeptidases. C-terminal amides cannot be produced by existing recombinant methods since the bacterial expression hosts lack the proper α-amidating enzyme (α-AE). This posttranslational modification is accomplished by an α-AE catalyzed O_2 oxidation of the C-terminal glycine of a Gly-extended precursor and subsequent decomposition of the α-hydroxyglycyl intermediate to the peptide amide and glyoxylate[81,82]:

$$\mathrm{RCONHCH_2CO_2^-} \xrightarrow[+1/2O_2]{\alpha\text{-AE}} \mathrm{RCONHCH(OH)CO_2^-} \xrightarrow[-OCH_2CO_2^-]{} \mathrm{RCONH_2} \quad (1)$$

We recently reported in vitro C-terminal amidations of synthetic Gly-extended GRF peptides by a purified recombinant α-AE.[83,84] The substrates, hGRF(1–44)-Gly-OH, hGRF(1–29)-Gly-OH and [Ala^{15}]-hGRF(1–29)-Gly-OH (prepared by solid-phase synthesis), were enzymatically converted to the respective products, hGRF(1–44)-NH_2, hGRF(1–29)-NH_2, and [Ala^{15}]-hGRF(1–29)-NH_2. Conversion to the amides by α-AE catalysis was nearly quantitative and practical yields of about 75% were obtained after preparative high performance liquid chromatography (HPLC) purification of the reaction mixtures. All the products gave satisfactory chemical analyses and were bioequivalent in vitro (growth hormone releasing activities and receptor binding affinities).

We also observed by HPLC the transient accumulation of an α-hydroxyglycyl peptide intermediate from the synthesis of the potent analogue [Ala^{15}]-hGRF(1–29)-NH_2, and isolated and characterized this product.[83] Using this purified α-hydroxyglycyl intermediate, we found that this recombinant α-AE was bifunctional, catalyzing both the oxidation and decomposition steps of the amidation process.

The existence of a suitable recombinant Gly-extended precursor for virtually any desired target peptide makes the α-AE method a general one and a natural complement to recombinant DNA methods. A large-scale synthesis of calcitonin from a recombinant Gly-extended precursor was recently reported that included methods for the production

of the recombinant enzyme.[85] Due to the relatively high cost and limited availability of α-AE, we also studied several alternate acyl-transfer routes to C-terminal amides utilizing readily available proteases.

Trypsin Catalyzed Direct Amidation. Our first attempt at protease catalyzed C-terminal amidation of an unprotected peptide was via direct amidation using ammonia as the nucleophile. Porcine trypsin was used to activate the -Arg^{29}-OH residue in [Ala^{15}]-hGRF(1–29)-OH and amidation was carried out in 1.4*M* NH_3/NH_4OAc buffer (pH 8.3) containing 95% (v/v) 1,4-butanediol cosolvent to form [Ala^{15}]-hGRF(1–29)-NH_2 (see Ref. 86):

$$[Ala^{15}]\text{-hGRF(1–29)-OH} + NH_3 \underset{\text{Trypsin}}{\overset{K_{syn}}{\rightleftharpoons}} [Ala^{15}]\text{-hGRF(1–29)-NH}_2 + H_2O \quad (2)$$

A stable equilibrium between the acid and amide [Eq. (2)] was established with $K_{syn} \approx 0.33$ (25% equilibrium yield). Attempts to drive this equilibrium further toward ammoniolysis by raising the pH or by adding more NH_3/NH_4OAc resulted in deactivation of trypsin. Trypsin catalysis was quite sluggish and hence large amounts of trypsin were needed to amidate a given amount of substrate in a reasonable length of time. The low trypsin activity is due partly to the use of a high content of cosolvent, which is a shortcoming suffered by many thermodynamically controlled systems. In addition, ammonia is an exceedingly poorly bound nucleophile for the protease.

An interesting aspect of the above direct amidation was that competing proteolytic degradations of the internal tryptic cleavage sites at Lys^{21}, Arg^{20}, Lys^{12}, and Arg^{11} were suppressed when the concentration of 1,4-butanediol cosolvent was ≥85% (v/v) and pH ≥ 8. The only side product detected under these conditions was a minor amount (3%) of [Ala^{15}]-hGRF(1–29)-$O(CH_2)_4OH$ from trypsin catalyzed acylation of the 1,4-butanediol cosolvent. This ester side product was isolated and identified by mass spectrometry.[86] Despite the disappointing yield and catalytic efficiency of this system, it was surprising and encouraging that an equilibrium was attained without trypsin severely degrading the peptide.

Trypsin Catalyzed Coupling of Leucine-Amide to a C-Terminal Acid Precursor of hGRF(1–44)-NH_2. Trypsin catalysis was also used to couple H-Leu-NH_2 to the -Arg^{43}-OH residue of the precursor hGRF(1–43)-OH to produce hGRF(1–44)-NH_2 (see Ref. 87):

$$\text{hGRF(1–43)-OH} + \text{H-Leu-NH}_2 \underset{\text{Trypsin}}{\rightleftharpoons} \text{hGRF(1–44)-NH}_2 + H_2O \quad (3)$$

Yields of 60–70% were obtained by using a large molar excess of H-Leu-NH_2 (1*M*) in 75% (v/v) N,N′-dimethylacetamide (DMAC) at room temperature. The overall catalytic activity of trypsin (synthetic and proteolytic) decreased gradually with an increasing % DMAC and then fell precipitously to undetectable levels at % DMAC > 76. This abrupt loss of activity was reversed by merely adding water to bring the % DMAC ≤ 76. The semisynthetic hGRF(1–44)-NH_2 was purified and shown to be chemically and biologically equivalent to a reference standard. The yield of hGRF(1–44)-NH_2 was limited by competing transpeptidations at Arg^{41} and Arg^{38} that gave [Leu^{42}]-hGRF(1–42)-NH_2 and [Leu^{39}]-hGRF(1–39)-NH_2 side products, respectively. As in the case of the trypsin catalyzed direct amidation, we were able to almost completely spur degradation of the remaining internal sites at Lys^{21}, Arg^{20}, Lys^{12}, and Arg^{11} by the use of cosolvents. The dibasic nature of the $Arg^{20}Lys^{21}$ and $Arg^{11}Lys^{12}$ sites may protect these tryptic points from proteolysis.

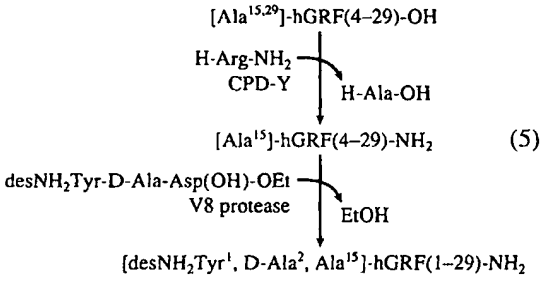
This system has several merits: The high solubility of both of the reactants under these conditions allows large amounts of the hGRF(1–43)-OH precursor to be processed in a relatively small total volume, thereby requiring less total H-Leu-NH_2 to obtain saturation ([H-Leu-NH_2] ≥ 1.0*M*), i.e., good economy of scale. The reaction occurs under mild conditions, i.e., room temperature at pH 5–8. The enzyme is relatively inexpensive, readily available, and efficient (at least 450 mg product/h/mg trypsin).

Carboxypeptidase Y Catalyzed Transpeptidation. Breddam and co-workers[88] recently developed a semisynthesis of hGRF(1–29)-NH_2 from the precursor [Ala^{29}]-hGRF(1–29)-OH via carboxypeptidase Y (CPD-Y) catalyzed exchange of the -Ala^{29}-OH residue for -Arg-NH_2:

$$[Ala^{29}]\text{-hGRF(1–29)-OH} + \text{H-Arg-NH}_2 \underset{\text{CPD-Y}}{\rightleftharpoons} \text{hGRF(1–29)-NH}_2 + \text{H-Ala-OH} \quad (4)$$

CPD-Y, an exoprotease from brewer's yeast, is well suited for this type of semisynthetic modification as it will not degrade internal peptide bonds as described above for endoproteases such as trypsin. -Ala[29]-OH was chosen as a favorable leaving group for this transpeptidation on the basis of model studies with the substrates Bzl-Met-Ser-X-OH (X = Ala, Leu, Arg). It was demonstrated that [Ala[29]]-hGRF(1–29)-OH (2 m*M*) could be converted to hGRF(1–29)-NH_2 (87% in 150 min) by CPD-Y catalysis in aqueous 1.5*M* H-Arg-NH_2 (pH 8).[90]

Two-Stage Enzymatic Semisynthesis of [desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH$_2$. We recently combined the above CPD-Y transpeptidation with a V8 protease catalyzed segment condensation to create a two-stage semisynthetic route[89,90] to the superpotent analogue, [desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, from a precursor potentially accessible by recombinant DNA synthesis, [Ala^{15,29}]-hGRF(4–29)-OH:



(5)

In the first stage the precursor, [Ala^{15,29}]-hGRF(4–29)-OH, was converted to the amidated segment, [Ala^{15}]-hGRF(4–29)-NH_2, by the method of Breddam et al.,[88] i.e., CPD-Y catalyzed transpeptidation in 1.0*M* H-Arg-NH_2. This CPD-Y catalyzed step was limited to conversions of 75–80% (62% isolated yield after HPLC purification) due to a competing hydrolysis to form the side product [Ala^{15}]-hGRF(4–28)-OH. The final product, [desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, was then prepared by V8 protease catalyzed kinetically controlled acylation of [Ala^{15}]-hGRF(4–29)-NH_2 using desNH$_2$Tyr-D-Ala-Asp(OH)-OEt in 20% (v/v) DMSO (pH 8.2, 37°C).[83] Conversion of [Ala^{15}]-hGRF(4–29)-NH_2 to [desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, using a 5-fold molar excess of the acyl component, desNH$_2$Tyr-D-Ala-Asp(OH)-OEt, was limited to 55–60% (49% isolated yield) due to competing proteolysis at Asp^{25}-Ile^{26} that generated several side products. The semisynthetic analogue was purified, fully characterized, and determined to possess the full biological activity of the chemically prepared standard.

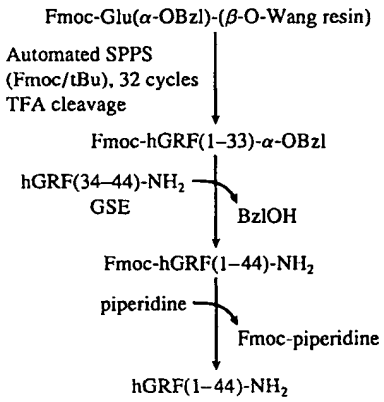
In the area of enzymatic peptide synthesis, it had previously been assumed that only "mildly activated" C-terminal esters (alkyl esters) were suitable enzyme substrates. Recently, Shellenberger et al.[91] reported that α-chymotrypsin catalyzed hydrolyses and aminolyses of the model system, maleyl-Leu-OR. These investigators demonstrated that the rate of hydrolysis or aminolysis varied with different ester functions (R = 4-$NO_2C_6H_4CH_2$ → $C_6H_5CH_2$ → CH_3-). This study prompted us to explore the use of the α-4-nitrobenzyl ester, desNH$_2$Tyr-D-Ala-Asp(OH)-ONb, in place of the original α-ethyl ester, which accelerated the V8 protease catalyzed segment condensation about 100-fold.[89,90] In addition to requiring much less enzyme, use of desNH$_2$Tyr-D-Ala-Asp(OH)-ONb resulted in a 90% conversion of [Ala^{15}]-hGRF(4–29)-NH_2 to the final product with no detectable loss of product to the slow proteolysis at Asp^{25}-Ile^{26}.

The synthesis was further improved[92,93] by replacing the V8 protease by the recently isolated Glu/Asp-specific endopeptidase (GSE) from *Bacillus licheniformis*,[94] which can be purified economically and in large scale from the commercial detergent Alcalase. Systematic studies of the kinetics of GSE catalyzed acylations of [Ala^{15}]-hGRF(4–29)-NH_2 were conducted utilizing a series of esters: desNH$_2$Tyr-D-Ala-Asp(OH)-OR [R = CH_3CH_2-, CH_3-, $ClCH_2CH_2$-, $C_6H_5CH_2$-, 4-$NO_2C_6H_4CH_2$-]. Rates of synthesis and hence the product yields varied as $C_6H_5CH_2$- > 4-$NO_2C_6H_4CH_2$- > $ClCH_2CH_2$- ⩾ CH_3- > CH_3CH_2-.[92] Quantitative conversion of [Ala^{15}]-hGRF(4–29)-NH_2 to [desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 could be obtained over several hours using an approximately 1000 : 1 (w/w) ratio of [Ala^{15}]-hGRF(4–29)-NH_2 to enzyme.

Convergent Solid-Phase/Enzymatic Synthesis of hGRF(1–44)-NH$_2$. It is known[94,95] that both the V8 protease and the GSE have a marked preference for Glu-Xaa sites over Asp-Xaa sites (ca. 1000–5000-fold). Thus the synthesis of Asp-Xaa bonds by these enzymes is probably only feasible for peptides, such as [desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, which contain Asp residues but no Glu residues. The synthesis of Glu-Xaa bonds by these enzymes, on the other hand, should be feasible for any peptide.

A convergent solid-phase/enzymatic synthesis of hGRF(1–44)-NH_2 employed a 33-mer + 11-mer GSE catalyzed segment condensation to form the

Glu^{33}-Ser^{34} bond [Eq. (6)]. This segment condensation involves competing proteolyses at Glu^{33}-Ser^{34}, Glu^{37}-Arg^{38}, Asp^{3}-Ala^{4}, and Asp^{25}-Ile^{26}. (Fmoc: 9-flourenylmethyoxycarbonyl; Bzl: benzyl; *t*Bu: *tert*-butyloxylcarbonyl; SPPS: solid-phase peptide synthesis.)



(6)

The acyl-donor segment, Fmoc-hGRF(1–33)-OBzl, was readily prepared by anchoring the side-chain carboxylate of Fmoc-Glu(OH)-OBzl to 4-alkoxybenzyl alcohol resin (Wang resin) followed by conventional automated SPPS with Fmoc/tBu-protected amino acids. The amino donor, hGRF(34–44)-NH_2, was also prepared by this route, starting with 4-(2,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetyl-benzhydrylamine resin.

The GSE catalyzed condensation of hGRF(34–44)-NH_2 and Fmoc-hGRF(1–33)-OBzl to produce Fmoc-hGRF(1–44)-NH_2 was performed in 60% v/v dimethylformamide, pH 8.8, 22°C. A 50% conversion of Fmoc-hGRF(1–33)-OBzl acyl donor to Fmoc-hGRF(1–44)-NH_2 in 200 min was observed by HPLC with most of the remaining acyl donor undergoing GSE catalyzed hydrolysis to Fmoc-hGRF(1–33)-OH (18%). The product was found to be identical to a reference standard by amino acid analysis, fast atom bombardment/mass spectrometry, and in vitro bioassay.

Natural Amino Acid Composition

A second strategy for linear GRF analogue development was to explore sequences consisting of only natural amino acid residues. The advantage of this strategy was largely economical, since nonamidated, natural amino acid sequence analogues could be produced on a large scale using current recombinant DNA methods. The feasibility of recombinant hGRF synthesis was rapidly demonstrated using both *E. coli* ([Leu^{27}]-hGRF(1–44)-OH: Ref. 46; [Ile^{27}]-hGRF(1–44)-OH and [Ile^{27}]-hGRF(1–29)-OH: Ref. 47; [Leu^{27}, Hse^{44}]-hGRF(1–44)-lactone: Ref. 96; [Leu^{27}, Hse^{45}]-bGRF(1–45)-lactone: Ref. 97) and *S. cerevisiae* ([Leu^{27}]-hGRF(1–40)-OH: Refs. 98 and 99) expression systems. However, none of these recombinant GRF analogues were exceedingly potent or resistant to enzyme catalyzed proteolysis. One initial objective of our laboratory was to determine the shortest nonamidated hGRF fragment that retained substantial biological activity. This was determined to be hGRF(1–32)-OH.

Initially applying Ala^{15}- and Leu^{27}- substitutions increased potency and resistance to oxidation[43,44] as expected, but did not address the issue of DPP-IV proteolysis. Therefore, natural amino acid replacements had to be found that conveyed DPP-IV resistance (the equivalent of $desNH_2Tyr^1$, D-Ala^2) yet did not detract from intrinsic activity. As mentioned previously (see section on enzymatic degradation pathways), the mouse GRF sequence was determined 7 years after that of hGRF and contained a unique His^1-Val^2 N-terminal.[11,12] Unlike all other known GRF sequences, mouse GRF proved to be surprisingly resistant to DPP-IV-like proteolysis in mouse or porcine plasma.[43,44] "Borrowing" from the mouse sequence, various [Val^2]-substituted hGRF(1–29)-NH_2,[56,57] bGRF(1–29)-NH_2,[100,101] and hGRF(1–32)-OH[43,44,57,100,101] analogues were synthesized and found to be resistant to plasma and purified DPP-IV degradation. Other position 2 replacements (e.g., Ile, Leu, Gly, Ser, Thr) were also found that conveyed resistance to enzymatic degradation (Table III),[56,57,100,102] although few were highly active in vitro (Table III)[102] and in vivo.[43,44,101–103] In combination with Ala^{15} and Leu^{27} substitution, the most potent natural sequence GRF analogue was obtained with Val^2 in swine[43,44] and either Val^2 or Ile^2 in cattle.[101,102,104]

It was most surprising to later discover that His^1 substitution (for Tyr^1) increased potency, improved DPP-IV resistance, and decreased plasma degradation in vitro (Tables II and III),[43,44,56,57] and produced superadditive effects with Val^2 in vivo.[43,44] This analogue, [His^1, Val^2, Ala^{15}, Leu^{27}]-hGRF(1–32)-OH, although potent, was not fully optimized as it retained Asn^8, which can deamidate/isomerize (see section on nonenzymatic degradation pathways). Again borrowing from rodent GRF sequences, Ser^8 (rat) and Thr^8 (mouse) replacements were explored, along with Gln^8 (not native to any known GRF), in an effort to reduce this nonenzymatic form of degradation. These replacements did indeed increase aqueous stability in vitro

Table III [Ala^{15}]-hGRF(1–29)-NH$_2$ Analogues with Natural N-Terminal Substitutions: Effects on Biological Activity and Resistance to Purified DPP-IV in vitro[a]

hGRF Analogue	Relative Potency[b]	Cleavage by Purified DPP-IV[c] (V_0/μmol min^{-1} mg^{-1})
hGRF(1–44)-NH$_2$	1.00	4.5
hGRF(1–29)-NH$_2$	0.71	5.0
[Ala^{15}]-hGRF(1–29)-NH$_2$	3.81	5.0
[His^1, Ala^{15}]-hGRF(1–29)-NH$_2$	3.03	3.0
[Leu^2, Ala^{15}]-hGRF(1–29)-NH$_2$	0.05	0.003
[Val^2, Ala^{15}]-hGRF(1–29)-NH$_2$	0.60	0.037
[Pro^2, Ala^{15}]-hGRF(1–29)-NH$_2$	0.82	9.8
[Ile^2, Ala^{15}]-hGRF(1–29)-NH$_2$	1.18	0.0
[Gly^2, Ala^{15}]-hGRF(1–29)-NH$_2$	2.26	1.4
[Ser^2, Ala^{15}]-hGRF(1–29)-NH$_2$	2.80	1.4
[His^1, Leu^2, Ala^{15}]-hGRF(1–29)-NH$_2$	0.03	0.0
[His^1, Ile^2, Ala^{15}]-hGRF(1–29)-NH$_2$	1.24	0.0
[His^1, Gly^2, Ala^{15}]-hGRF(1–29)-NH$_2$	1.86	0.3
[His^1, Val^2, Ala^{15}]-hGRF(1–29)-NH$_2$	2.95	0.0

[a] Data in part from Bongers et al.[56] and Heimer et al.[57]

[b] GH-releasing potency relative to hGRF(1–44)-NH$_2$; determined using cultured rat pituitary cells exposed to 3.1 → 400 pM hGRF analogue for 4 h, 37°C.

[c] Incubation with human placental DPP-IV (0.52 μg/mL, pH 7.8, 37°C). An initial velocity of 0 indicates no proteolysis over 12–24 h of incubation.

(Table IV) and markedly enhanced in vivo potency (11–13-fold over hGRF) when inserted into the [His^1, Val^2, Ala^{15}, Leu^{27}]-hGRF(1–32)-OH template.[43,44]

The practicality of synthesizing a potent, long-acting, natural sequence analogue by wholly recombinant means has recently been realized. [His^1, Val^2, Gln^8, Ala^{15}, Leu^{27}]-hGRF(1–32)-OH has

Table IV In vitro Assays of Natural Sequence hGRF(1–32)-OH (hGRF$_{32}$) Analogue[a]

hGRF Analogue	Relative Potency[b]	Aqueous Stability[c] $T_{1/2}$ (h)	Plasma Stability[d] $T_{1/2}$ (min)
hGRF(1–44)-NH$_2$	1.0	76	7.4
[Ala^{15}]-hGRF$_{32}$	2.1	139	8.0
[Ala^{15}, Leu^{27}]-hGRF$_{32}$	1.9	121	15
[His^1, Ala^{15}, Leu^{27}]-hGRF$_{32}$	3.0	145	25
[Val^2, Ala^{15}, Leu^{27}]-hGRF$_{32}$	0.3	130	35
[His^1, Val^2, Ala^{15}, Leu^{27}]-hGRF$_{32}$	2.8	208	45
[His^1, Val^2, Ser^8, Ala^{15}, Leu^{27}]-hGRF$_{32}$	3.1	1470	42
[His^1, Val^2, Thr^8, Ala^{15}, Leu^{27}]-hGRF$_{32}$	2.5	1046	49
[His^1, Val^2, Gln^8, Ala^{15}, Leu^{27}]-hGRF$_{32}$	2.8	826	43

[a] Data from Campbell et al.[44]

[b] GH-relesing potency relative to hGRF(1–44)-NH$_2$; determined using cultured rat pituitary cells exposed to 3.1 → 400 pM hGRF analogue for 4 h, 37°C.

[c] The 0.5 mg/mL analogue incubated in 0.25*M* Na_2HPO_4/H_3PO_4 at 37°C (pH 7.4); peptide content of aliquots determined by analytical HPLC. Half-life ($T_{1/2}$) was determined by rat of disappearance.

[d] The 0.1 mg/mL GRF analogue incubated in porcine plasma at 37°C and aliquots extracted using C_{18} SEP-PAK columns; peptide content determined by analytical HPLC. Half-life ($T_{1/2}$) was determined by rate of disappearance.

been recombinantly produced on a gm scale in *E. coli* using a fusion protein (J. Smart, H. Oppermann, and R. Tucker, unpublished observations). Interestingly enough, the fusion protein may be cleaved enzymatically using either V8 protease or GSE.

CYCLIC GRF ANALOGUES

Rationale for GRF Cyclization

The introduction of constraints into peptides and proteins is known to reduce the number of possible conformations. Incorporation of constraints into biologically active peptides may result in rigidified analogues. Such analogues are expected to fulfill the necessary conformational requirements for improved receptor binding and may have enhanced or prolonged biological activity.[105] Of the various methods that are available for preparing constrained peptides, cyclization of the biologically active lead linear peptides are a major target since the cyclic peptides are expected to adopt conformations that are more well defined than their linear counterparts. Cyclic peptides have also been shown to be more resistant to enzymatic and proteolytic degradation and to have increased hydrophobicity that may improve their absorption.

Peptides may be cyclized through a variety of functionalities (e.g., cystine bridged, lactones, lactams) and at various positions (N-terminus to C-terminus; C-terminus to side chain; N-terminus to side chain; N-backbone to N-terminus; N-backbone to C-terminus or side chain to side chain). Recent progress in the solid-phase synthesis of cyclic peptides, including the development of novel solid supports and orthogonal protecting groups and a new generation of cyclization reagents [e.g., benzotriazol-1-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and HBTU], prompted us to focus on the synthesis of novel side-chain to side-chain cyclic (lactam) GRF analogues.

Monocyclic GRF Analogues

As described earlier, the replacement of Gly^{15} by Ala^{15} in GRF results in analogues with enhanced biological activity, which is attributed to increased α-helicity and maximization of amphiphilic character. In addition to conformers such as these with a single straight helical segment, there is another family of conformers that form helices with kinks near residues 16 and/or 25. These initial observations laid important groundwork for the synthesis of novel side-chain to side-chain cyclic (lactam) analogues of GRF that were designed to stabilize the α-helix at various $i - (i + 4)$ positions in the backbone. This spacing is the same as that required for stabilization of an α-helical conformation for salt-bridge formation between Glu and Lys residues in a series of peptides in which these positions were varied.[106] Relatively little information is known about the optimal geometry for salt-bridge formation or optimal ring sizes for maximization of α-helicity.

Molecular modeling predicts that lactamization of the $i - (i + 4)$ positions in the backbone should cause minimal disruption of the α-helical conformation. The GRF system is particularly well suited to these side-chain to side-chain cyclizations since the parent peptide contains several pairs of Asp-Lys residues that retain the $i - (i + 4)$ relationship. Our primary studies therefore focused on the synthesis of substituted cycloGRF(1–29)-NH_2, which contained the replacements (i.e., desNH$_2$Tyr^1, D-Ala^2, Ala^{15}) that were found to improve biological potency in the linear GRF system.[17,20,27] In order to synthesize these cyclic analogues, we developed a procedure using an orthogonal protection scheme using the Boc/Bzl strategy with -OFm and Fmoc- side-chain protection for Asp/Glu and Lys, respectively.[107] Following selective deprotection of the Fmoc-based protecting groups, BOP[104] was found to be a preferred reagent for the side-chain to side-chain solid phase cyclization.[107]

Our initial studies confirmed the importance of retaining the $i - (i + 4)$ side-chain to side-chain lactam bridge since several non-$i - (i + 4)$ GRF analogues [e.g., cyclo(Asp^3-Lys^{12})-, cyclo(Lys^{11}-Glu^{16})-, and cyclo(Glu^{16}-Lys^{21})-] were almost devoid of biological activity (Table V) and possessed substantial decreases in α-helicity as determined from their CD spectra (220 and 208 nm Cotton Effects).[27] Studies on a series of $i - (i + 4)$ cyclic analogues of GRF (Table V) revealed that cyclo(Lys^4-Asp^8)-, cyclo(Asp^8-Lys^{12})-, cyclo(Lys^{12}-Glu^{16})-, and cyclo(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH_2 retained significant levels of in vitro potency.[108] CD studies demonstrated that these $i - (i + 4)$ cyclic analogues retained substantial segments of α-helicity.

Structure–activity studies led to the conclusion that the trisubstituted analogues, cyclo(Asp^8-Lys^{12})-[desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 and cyclo(Lys^{21}-Asp^{25})-[desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, were the most active cy-

Table V Biological Activity of Cyclic hGRF(1–29)-NH_2 ($hGRF_{29}$) Analogues

Type	hGRF Analogue	Relative Potency[a]
Linear	hGRF(1–44)-NH_2	1.00
	[Ala^{15}]-$hGRF_{29}$	3.81
$i-(i+9)$	Cyclo(Asp^3-Lys^{12})-[Ala^{15}]-$hGRF_{29}$	0.07
$i-(i+5)$	Cyclo(Lys^{11}-Glu^{16})-[Ala^{15}]-$hGRF_{29}$	0.02
$i-(i+5)$	Cyclo(Glu^{16}-Lys^{21})-[Ala^{15}]-$hGRF_{29}$	0.02
$i-(i+4)$	Cyclo(Lys^4-Asp^8)-[Ala^{15}]-$hGRF_{29}$	1.58
	Cyclo(Asp^8-Lys^{12})-[Ala^{15}]-$hGRF_{29}$	0.77
	Cyclo(Lys^{12}-Glu^{16})-[Ala^{15}]-$hGRF_{29}$	0.80
	Cyclo(Glu^{16}-Lys^{20})-[Ala^{15}]-$hGRF_{29}$	0.24
	Cyclo(Lys^{21}-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	1.33
	Cyclo(Asp^8-Lys^{12})-[$desNH_2Tyr^1$, D-Ala^2, Ala^{15}]-$hGRF_{29}$	2.47
	Cyclo(Lys^{21}-Asp^{25})-[$desNH_2Tyr^1$, D-Ala^2, Ala^{15}]-$hGRF_{29}$	1.33
Multiple $i-(i=4)$	Dicyclo(Asp^8-Lys^{12})(Glu^{16}-Lys^{20})-[Ala^{15}]-$hGRF_{29}$	0.02
	Dicyclo(Glu^{16}-Lys^{20})(Lys^{21}-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	0.02
	Dicyclo(Lys^{12}-Glu^{16})(Lys^{21}-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	0.23
	Dicyclo(Asp^8-Lys^{12})(Lys^{21}-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	0.69
	Dicyclo(Asp^8-Lys^{12})(Lys^{21}-Asp^{25})-[$desNH_2Tyr^1$, D-Ala^2, Ala^{15}]-$hGRF_{29}$	2.81
	Dicyclo(Asp^8-[Gly]-Orn^{12})(Orn^{21}-[Gly]-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	4.36
	Tricyclo(Asp^8-Lys^{12})(Glu^{16}-Lys^{20})(Lys^{21}-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	< 0.01

[a] GH-releasing potency relative to hGRF(1–44)-NH_2; determined using cultured rat pituitary cells exposed to 3.1 → 400 pM hGRF analogue for 4 h, 37°C.

clic analogues in the series, although somewhat less active than the corresponding linear peptides. Molecular dynamics calculations, based on NOE-derived distance constraints, demonstrated that the lactam formed between the side chains of Asp^8-Lys^{12} and Lys^{21}-Asp^{25} stabilizes the α-helical conformation of the peptide backbone (Figure 4A and B) and is consistent with our conclusion that GRF binds to its receptor in a helical conformation.[70] However, stabilization of the α-helix is accompanied by slightly reduced biological activity. From these observations it was concluded that the lactam may prevent proper orientation of all the side chains that interact with the receptor or it may hinder the formation of the kink in the α-helix that was observed for the linear GRF analogues and that may be required for optimal binding.

Monocyclic GRF Analogues: Variation of Lactam Ring Size

The $i-(i+4)$ side-chain to side-chain lactam bridges in the biologically active cycloGRF analogues described above are all 20-member rings (i.e., Asp^i-Lys^{i+4} and Lys^i-Asp^{i+4}). Studies were undertaken to determine the optimal ring size for biological activity and two series of cyclic $i-(i+4)$ homologues of GRF ranging from 18- through 24-member rings were prepared corresponding to $cyclo^{8,12}$- and $cyclo^{21,25}$-[Ala^{15}]-hGRF(1–29)-NH_2. Cyclic homologues containing 18–20-member rings were prepared by the procedure described above (Boc/Bzl strategy with -OFm and Fmoc- protection for the side-chain sites to be cyclized). However, homologues containing 21–24 member rings required the introduction of "spacer" residues (e.g., Gly, β-Ala or γ-Aba) as depicted in Figure 5(A) and 5(B) corresponding, respectively, to extended (N- to C-terminal) and reverse-extended (C- to N-terminal) lactam ring systems. Preliminary results on the structures of the GRF analogues containing both extended and reverse-extended lactam ring structures using spacer residues (Gly, β-Ala, and γ-Aba) have been reported by our group.[109] Other intrachain branched cyclic peptides containing N-terminal extended lactam ring systems have recently been reported for a family of α-MSH structures[110] and for a human red cell anion transporter band peptide.[111]

We have developed general procedures for extending the lactam ring system utilizing the above Boc/Bzl solid-phase strategy to include the inser-

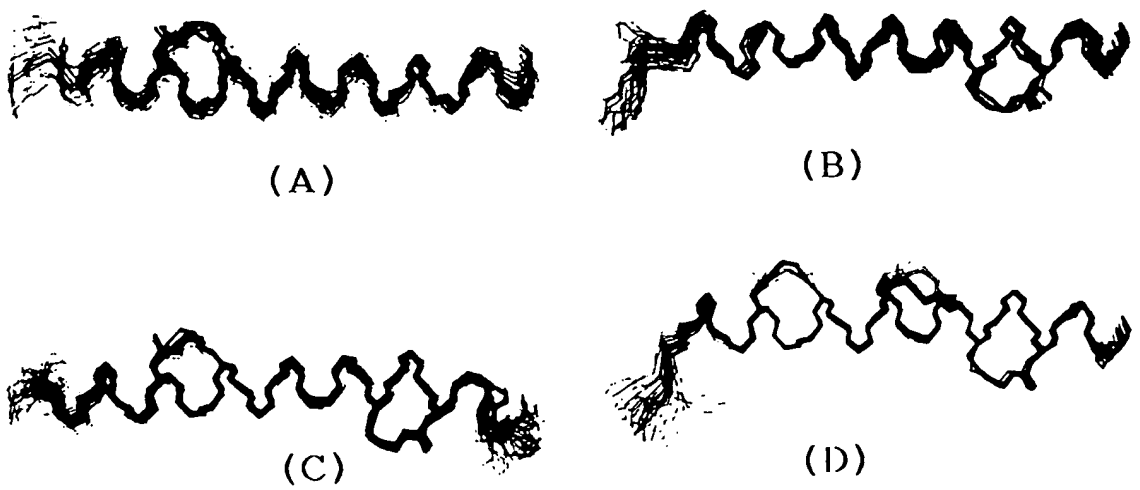


FIGURE 4 Superposition of structures of the GRF analogues obtained from evaluation of the nmr data in 75% methanol, pH 6.0, by constrained dynamics and minimization calculations. Structures are oriented with the N-terminus toward the left. (A) Cyclo(Asp^{8}-Lys^{12})-[Ala^{15}]-hGRF(1–29)-NH$_2$. (B) Cyclo(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH$_2$. (C) Dicyclo(Asp^{8}-Lys^{12})(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH$_2$. (D) Tricyclo(Asp^{8}-Lys^{12})(Glu^{16}-Lys^{20})(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH$_2$.

tion of "spacer" amino acids between the side chains of basic (e.g., Lys, Orn, Dpr) and acidic (e.g., Asp, Glu) residues as outlined in Figures 6 and 7. The extended lactam ring system, corresponding to cyclo^{8,12}-homologues in the GRF system, required the insertion of "spacer" Fmoc-amino acids coupled to the side chain of the basic residue at position 12 (Figure 6). The reverse-extended lactam ring system, corresponding to cyclo^{21,25}-homologues in the GRF system, was carried out by either of the two strategies displayed in Figure 7. The first strategy (Figure 7, left) is similar to that used for the extended lactams except that fluorenylmethyl ester spacers were coupled to the side chain of the acidic residue at position 25. These fluorenylmethyl esters were prepared in two steps by (a) DCC-induced esterification of Boc-Gly-OH (Boc-β-Ala-OH or Boc-γ-Aba-OH) with fluorenylmethyl alcohol followed by (b) selective removal of the Boc-protecting group with HCl in dioxane.[112] Alternatively, a synthesis was developed (Figure 7, right) that utilized an orthogonal solid-phase strategy including

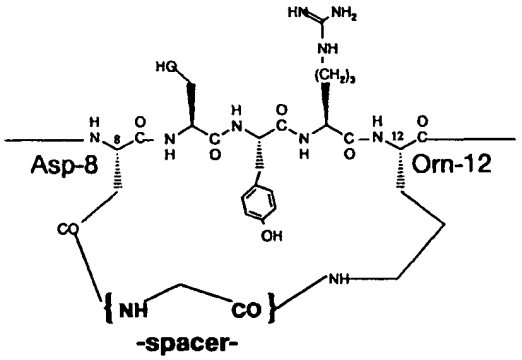


FIGURE 5A Structure of the extended 22-membered $i-(i+4)$ cyclic lactam ring system showing the position of the glycine spacer residue between Asp^{8} and Orn^{12}.

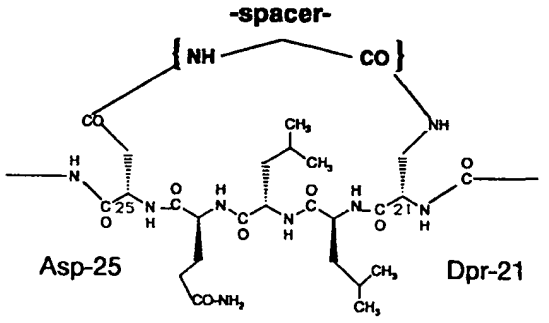


FIGURE 5B Structure of the reverse-extended 20-membered $i-(i+4)$ cyclic lactam ring system showing the position of the glycine spacer between Dpr^{21} and Asp^{25}.

allyl ester protection of the acidic residue (e.g., Asp or Glu), which enabled selective deprotection of the Fmoc-protecting group at the basic residue (position 21) and the insertion of the spacer amino acid at this site via the use of Fmoc-amino acids. After removal of the side-chain protecting groups, lactamization was carried out by the BOP procedure.[27]

The in vitro biological activity of the $i - (i + 4)$ homologues of cyclo^{8,12}- and cyclo^{21,25}-[Ala^{15}]-hGRF(1–29)-NH_2 are summarized in Table V. The ring size was varied from 18 through 24 members. In order to confirm that the observed biological activity was due to ring size rather than position of the amide function in the lactam bridge, several variations were incorporated for the 20-, 21-, and 22-member rings. In each case the in vitro potency as well as the α-helicity was unaffected by the position of the amide fu in the lactam bridge. Although the relation between ring size and biological activity was remarkably similar for both the cyclo^{8,12}- and cyclo^{21,25}-[Ala^{15}]-hGRF(1–29)-NH_2,

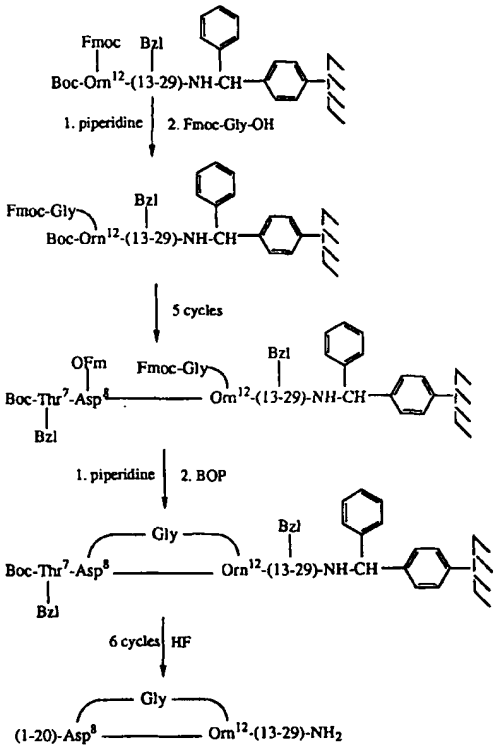


FIGURE 6 Synthetic scheme for the synthesis of the 22-membered $i - (i + 4)$ extended lactam ring system.

the biological activity of these cyclic peptides was very dependent upon the ring size. The GRF analogues with constrained rings ($\leq$19 members) are biologically inactive in both series. The most active homologues in the cyclo^{8,12}-[Ala^{15}]-hGRF(1–29)-NH_2 series have lactam rings with 21 and 22 members. Homologues with smaller (20 members) and larger rings (23 and 24 members) retained substantial biological activity. The most active homologues in the cyclo^{21,25}-[Ala^{15}]-hGRF(1–29)-NH_2 series have lactam rings with 22 members; homologues with smaller (20 and 21 members) and larger (23 and 24 members) also retained substantial levels of in vitro potency. Conformational analysis (CD studies) reveals that the biologically active peptides in both series (20–24 members) retain more α-helicity than the biologically inactive homologues possessing $\leq$19-member rings that partially destabilize the α-helix (Figures 8 and 9). Interestingly, molecular dynamics studies on the highly potent 22-member lactam, cyclo(Asp^8-[Gly]-Orn^{12})-[Ala^{15}]-hGRF(1–29)-NH_2, revealed some loss of α-helicity resulting from extensive fraying at the COOH-terminus. This observation was unexpected, and the high potency of this peptide is rationalized on the basis of the flexibility of the 22-member ring, which may be sufficiently flexible to assume the required conformation at the receptor.

Multicyclic GRF Analogues

The solid-phase procedures that were designed for the synthesis of the monocyclic analogues of GRF (Boc/Bzl strategy with -OFm and Fmoc- protection for the side-chain sites to be cyclized) were modified to prepare of a series of multiple $i - (i + 4)$ analogues of GRF that possess both extended and reverse-extended lactams.[113] Dicyclo(Lys^{12}-Glu^{16})(Lys^{21}-Asp^{25})-, dicyclo(Asp^8-Lys^{12})(Glu^{16}-Lys^{20})-, and dicyclo(Glu^{16}-Lys^{20})(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH_2 were less potent than dicyclo(Asp^8-Lys^{12})(Lys^{21}-Asp^{25})-Ala^{15}]-hGRF(1–29)-NH_2, which contains two 20-member lactam rings and was nearly equipotent in vitro to the corresponding monocyclic GRF analogues: cyclo(Asp^8-Lys^{12})-[Ala^{15}]-hGRF(1–29)-NH_2 and cyclo(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH_2. The trisubstituted dicyclo-analogue, dicyclo(Asp^8-Lys^{12})(Lys^{21}-Asp^{25})-[desNH$_2$Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, also had the same in vitro potency as the corresponding monocyclic peptides. The results of conformational studies (molecular dynamics calculations, based on NOE-derived

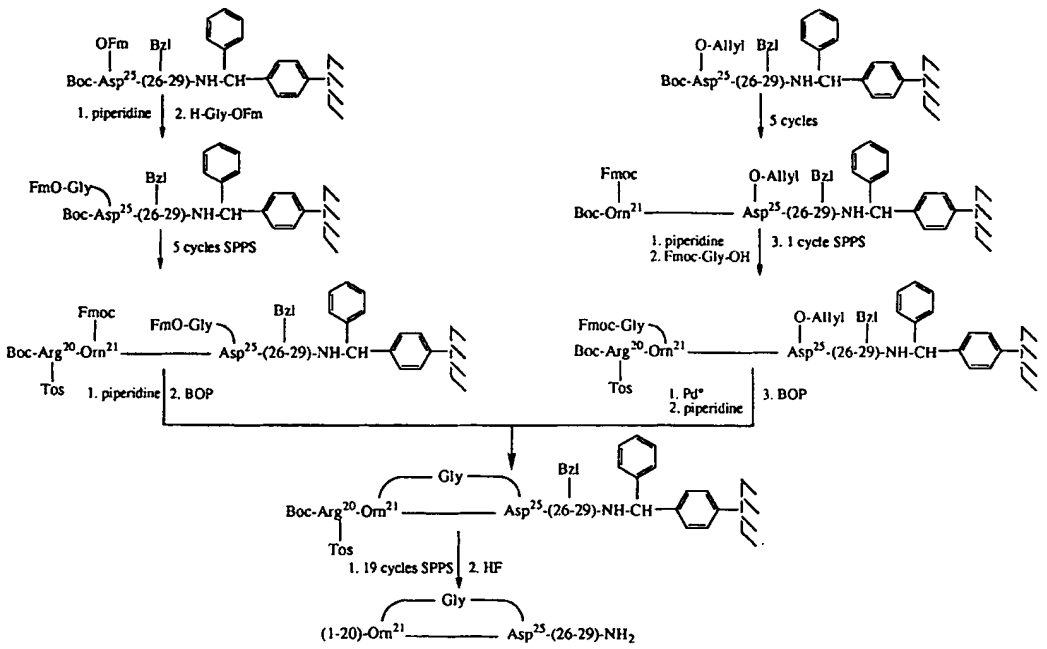


FIGURE 7 Alternate synthetic schemes for the synthesis of the 22-membered $i - (i + 4)$ reverse-extended lactam ring system. Left: Incorporation of the glycine spacer via insertion of glycine-fluorenylmethyl ester to the side chain of the Asp residue at position 25. Right: Incorporation of the glycine spacer via insertion of Fmoc-glycine to the side chain of the Orn residue at position 21.

distance constraints) of dicyclo(Asp^8-Lys^{12})(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH_2 is shown in Figure 3C and revealed an N-terminal helical segment spanning residues 7–20, but its C-terminal helical segment was not well ordered.[70] Another dicyclo-hGRF analogue, dicyclo(Asp^8-[Gly]-Orn^{12})-Orn^8-[Gly]-Asp^{12})-hGRF(1–29)-NH_2, which contains two "optimum" 22-member lactam rings, was found to be the most active multiple cyclic peptide in the series. Finally, tricyclo(Asp^8-Lys^{12})(Glu^{16}-Lys^{20})(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH_2 was synthesized by the same general procedures and found to retain substantial α-helicity but was inactive in vitro. Molecular dynamics calculations on the tricyclic peptide (Figure 4D) revealed that it was in a rigid helical conformation. It was concluded that rigidification of the central α-helix may hinder the backbone from assuming a required conformation for optimal receptor binding.

As mentioned earlier, an important aspect of these cyclic analogues is their increased resistance to enzymatic degradation (e.g., DPP-IV and trypsin) and therefore their longer $t_{1/2}$ in vivo. Our studies revealed that native GRF(1–44)-NH_2 undergoes 65% degradation after incubation in human plasma for 60 min at 37°C. Cyclo(Asp^8-Lys^{12})-[Ala^{15}]-hGRF(1–29)-NH_2 and cyclo(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH_2 were much more stable to plasma degradation undergoing, respectively, 12 and 23% degradation. Under the same conditions, dicyclo(Asp^8-Lys^{12})(Lys^{21}-Asp^{25})-[Ala^{15}]-hGRF(1–29)-NH_2 was only 5% degraded after incubation in human plasma.[70] The enhanced stability of the cyclic GRF analogues to enzymatic degradation was attributed to the effect of the lactam ring on stabilizing the helical conformation near the NH_2-terminus and disrupting interaction with the degrading enzyme (DPP-IV).

Biological Activity of Cyclic GRF Analogues

Cyclic analogues of hGRF possessing $i - (i + 4)$ lactam rings at various positions throughout the backbone of [Ala^{15}]-hGRF(1–29)-NH_2 were prepared to study the effects of α-helical stabilization on biological activity. The cyclo^{4,8}-, cyclo^{8,12}-,

Table VI Biological Activity of Cyclic hGRF(1–29)-NH_2 ($hGRF_{29}$) Analogues with Varying Ring Size

Type	hGRF Analogue	Ring Size	Relative Potency[a]
$Cyclo^{8,12}$	Cyclo(Asp^8-[γ-Aba]-Orn^{12})-[Ala^{15}]-$hGRF_{29}$	24	1.42
	Cyclo(Glu^8-[Gly]-Orn^{12})-[Ala^{15}]-$hGRF_{29}$	23	1.68
	Cyclo(Asp^8-[Gly]-Orn^{12})-[Ala^{15}]-$hGRF_{29}$	22	4.90
	Cyclo(Glu^8-[β-Ala]-Dpr^{12})-[Ala^{15}]-$hGRF_{29}$	22	3.79
	Cyclo(Glu^8-Lys^{12})-[Ala^{15}]-$hGRF_{29}$	21	3.43
	Cyclo(Glu^8-[Gly]-Dpr^{12})-[Ala^{15}]-$hGRF_{29}$	21	3.80
	Cyclo(Asp^8-Lys^{12})-[Ala^{15}]-$hGRF_{29}$	20	0.77
	Cyclo(Glu^8-Orn^{12})-[Ala^{15}]-$hGRF_{29}$	20	1.77
	Cyclo(Asp^8-[Gly]-Dpr^{12})-[Ala^{15}]-$hGRF_{29}$	20	1.79
	Cyclo(Asp^8-Orn^{12})-[Ala^{15}]-$hGRF_{29}$	19	0.03
	Cyclo(Glu^8-Dpr^{12})-[Ala^{15}]-$hGRF_{29}$	18	0.02
$Cyclo^{21,25}$	Cyclo(Orn^{21}-[γ-Aba]-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	24	2.68
	Cyclo(Orn^{21}-[Gly]-Glu^{25})-[Ala^{15}]-$hGRF_{29}$	23	2.53
	Cyclo(Orn^{21}-[Gly]-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	22	4.14
	Cyclo(Dpr^{21}-[β-Ala]-Glu^{25})-[Ala^{15}]-$hGRF_{29}$	22	4.05
	Cyclo(Lys^{21}-Glu^{25})-[Ala^{15}]-$hGRF_{29}$	21	2.41
	Cyclo(Dpr^{21}-[Gly]-Glu^{25})-[Ala^{15}]-$hGRF_{29}$	21	2.69
	Cyclo(Lys^{21}-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	20	1.33
	Cyclo(Orn^{21}-Glu^{25})-[Ala^{15}]-$hGRF_{29}$	20	0.81
	Cyclo(Dpr^{21}-[Gly]-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	20	1.73
	Cyclo(Orn^{21}-Asp^{25})-[Ala^{15}]-$hGRF_{29}$	19	0.04
	Cyclo(Dpr^{21}-Glu^{25})-[Ala^{15}]-$hGRF_{29}$	18	0.03

[a] GH-releasing potency relative to hGRF(1–44)-NH_2; determined using cultured rat pituitary cells exposed to 3.1 → 400 pM hGRF analogue for 4 h, 37°C.

$cyclo^{12,16}$-, and $cyclo^{21,25}$- systems all retained significant levels of in vitro biological activity (Table V). Conformational analysis demonstrated a good correspondence between α-helical stabilization and biological activity, although rigidification of the central helical region was accompanied by slightly reduced potency. It is proposed that a kink observed in the linear peptide, [Ala^{15}]-hGRF(1–29)-NH_2, may be important for optimal receptor binding. The cyclic peptides were much more stable to plasma degradation than the corresponding linear analogue. An in vivo study in swine using cyclo(Asp^8-Lys^{12})-[$desNH_2Tyr^1$, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 was approximately 10–20-fold more potent than native GRF(1–44)-NH_2.[114] Synthetic procedures have been developed for extending the solid phase (Boc/Bzl) strategy to include the synthesis of cyclic peptides possessing both extended and reverse-extended lactams using spacer residues (e.g., Gly, β-Ala, γ-Aba). Using these procedures, two series of cyclic $i - (i + 4)$ GRF analogues were synthesized to determine the optimum ring size for maximum biological activity. Optimal ring size for both series of cyclic GRF analogues was determined to be 22 members (Table VI). Although cyclic GRF analogues containing larger (23–24 members) and smaller rings (20–21 members) retain substantial potencies, peptides with constrained rings (≤19 members) are biologically inactive and have partially destabilized α-helical conformations.

SUMMARY

In the 12 years following the sequencing of hGRF, substantial efforts have been expended in attempts to produce potent, long-acting GRF analogues. These efforts have produced novel linear (natural and "unnatural" sequence) and cyclic analogues. One such linear, unnatural sequence hGRF analog, [$desNH_2Tyr^1$, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, was designed on the basis of improved receptor affinity (Ala^{15} substitution), GH-releasing activity (Ala^{15} substitution), and DPP-IV resistance ($desNH_2Tyr^1$-D-Ala^2 substitution) in vitro. The enhanced in vitro profile of [$desNH_2Tyr^1$, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 translated into a 10–15-fold increase in biological potency in vivo. Confor-

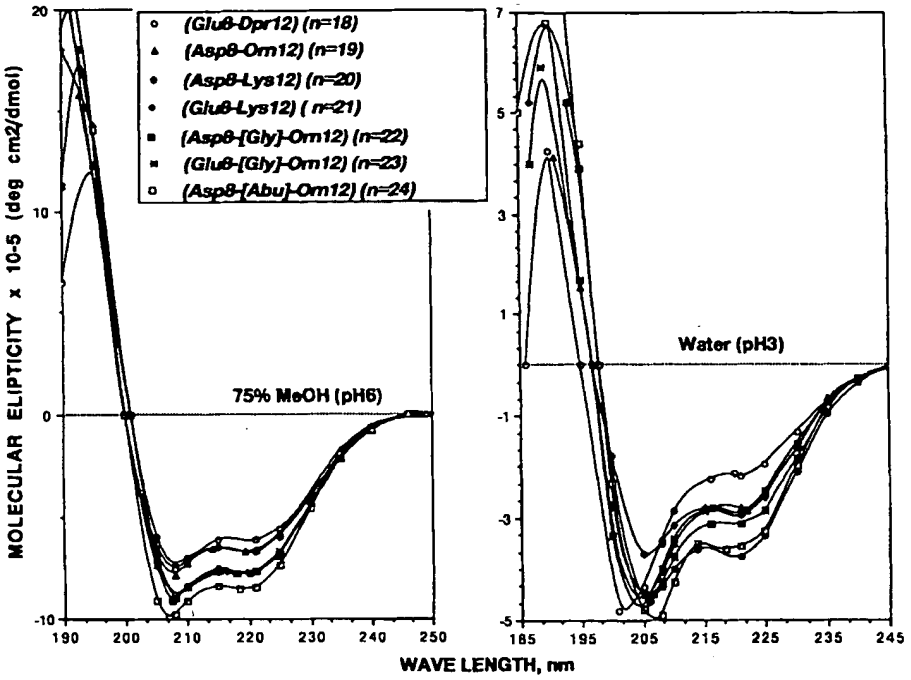


FIGURE 8 CD of the cyclo^{8,12}-[Ala^{15}]-hGRF(1–29)-NH_2 family of peptides with variation of the lactam ring size from n = 18 through n = 24 members. Left: Spectra in 75% methanol, pH 6. Right: Spectra in water, pH 3.

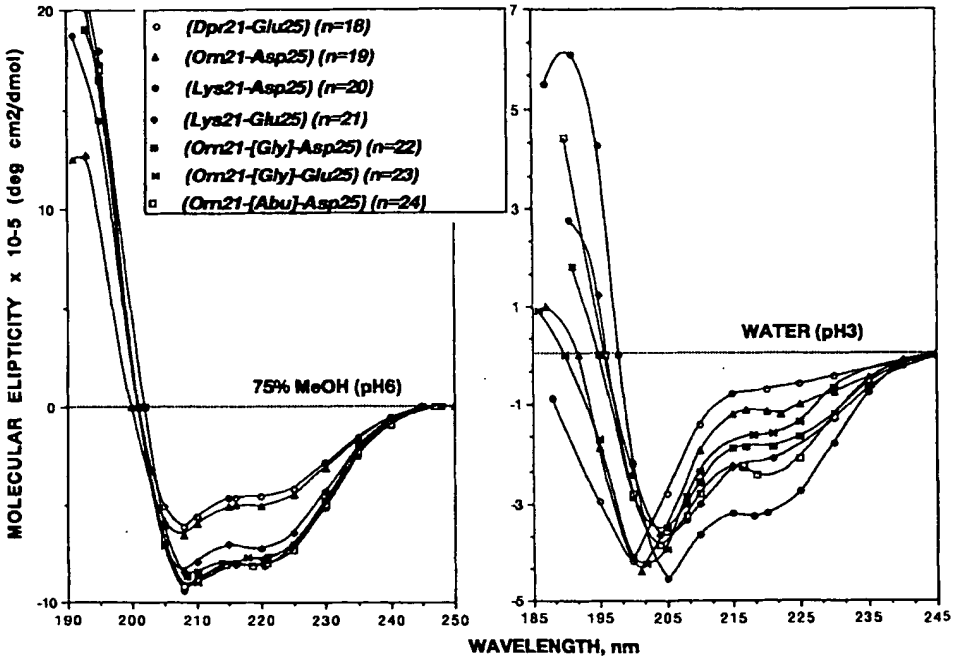


FIGURE 9 CD of the cyclo^{21,25}-[Ala^{15}]-hGRF(1–29)-NH_2 family of peptides with variation of the lactam ring size from n = 18 through n = 24 members. Left: Spectra in 75% methanol, pH 6. Right: Spectra in water, pH 3.

mational studies on potent linear analogues, e.g., [Ala^{15}]-hGRF(1–29)-NH_2 and [desNH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2, led to an important correlation between biological activity, increased α-helicity, and maximization of amphiphilic character. From these observations, cyclic analogues of hGRF were designed to simulate and constrain the α-helical conformation. Spacers were inserted, where necessary, to optimize lactam ring size (21–22 members preferred). Of these cyclic hGRF analogues, cyclo(Asp^8-[Gly]-Orn^{12})-[Ala^{15}]-hGRF(1–29)-NH_2 (22-member lactam) and cyclo (Orn^{21}-[Gly]-Asp^{21})-[Ala^{15}]-hGRF(1–29)-NH_2 (22-member lactam) were found to be 4–5-fold more potent than hGRF in vitro.

Several methods of enzyme catalyzed syntheses of hGRF and analogues were studied to develop economic production methods. For the native hGRF or hGRF(1–29)-NH_2 fragment (human clinical application), the most favorable enzymatic process would likely be via recombinant production of hGRF(1–44)-Gly-OH or hGRF(1–29)-Gly-OH, respectively, and subsequent amidation by the α-AE. In the case of the potent analogue, [des-NH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 (human or veterinary application), the most practical procedure involves (1) solution-phase synthesis of desNH_2Tyr-D-Ala-Asp(OH)-OEt, (2) recombinant synthesis of [Ala^{15,29}]-hGRF(1–29)-OH, and (3) enzymatic amidation with CPD-Y and segment coupling with GSE ("one-pot synthesis"). It is apparent that many of these analogues (e.g., linear unnatural sequence and cyclic) are hampered by current economics from finding practical application. It is likely that this situation will change as enzymes become less expensive and/or synthetic strategies become more efficient. The wholly recombinant production of potent ($\approx$ 13-fold greater activity than hGRF in vivo), natural sequence analogues such as [His^1, Val^2, Gln^8, Ala^{15}, Leu^{27}]-hGRF(1–32)-OH is currently the most promising. This natural sequence analogue was an improvement upon [desNH_2Tyr^1, D-Ala^2, Ala^{15}]-hGRF(1–29)-NH_2 as it was more chemically stable (i.e., resistant to Met^{27} oxidation and Asn^8 deamidation) and more economical to produce in large scale. As such, [His^1, Val^2, Gln^8, Ala^{15}, Leu^{27}]-hGRF(1–32)-OH has enormous potential for use in human (treatment of pituitary dwarfism) and veterinary (performance enhancement) applications.

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